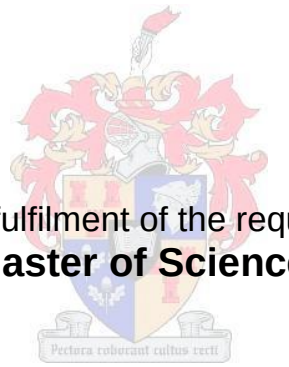
No Evidence of Metabolic Cold Adaptation in *Drosophila melanogaster* Along a Latitudinal Gradient in Australia

by

Sinéad Erin O'Toole



Thesis presented in partial fulfilment of the requirements for the degree of
Master of Science

at

Stellenbosch University
Department of Conservation Ecology and Entomology, Faculty of AgriSciences

Supervisor: Prof. John Terblanche
Co-supervisor: Prof. Steven Chown
Co-supervisor: Dr. Carla Sgrò

March 2018

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 20/11/2017

Summary

Metabolic cold adaptation (MCA) is a controversial hypothesis that suggests cold-adapted species should have a higher resting metabolic rate (RMR), or an altered metabolic rate- temperature (MR-T) relationship when compared to their warmer counterparts at the same test temperature. This intraspecific study provides a novel experimental assessment testing MCA in four populations of *Drosophila melanogaster* found across a latitudinal cline of eastern Australia. Specifically, I test the prediction that the higher latitude populations (from colder environmental temperatures) show an elevated RMR or a lower/higher activation energy (E_a) which might reflect a change in MR-T sensitivity. Populations were reared in the laboratory under common conditions before being acclimated for 5 days to stable 18°C and 25°C, as well as fluctuating temperatures of 13-22°C and 20-30°C to test if MR was affected by acclimation. MR was estimated at 6 test temperatures (10°C, 15°C, 20°C, 25°C 30°C and 35°C). After adjusting for mass and activity, no population was found to have a consistently higher RMR at any acclimation condition. Acclimation to stable 25°C resulted in slightly lower E_a for two populations, but decreasing E_a did not correlate with increasing latitude. No other differences in E_a were found. The study concludes there is no evidence of MCA among populations of *D. melanogaster* in Australia- possibly due to the lack of consistently cold temperatures in some higher latitudes of the Southern Hemisphere that would perhaps be likely to drive such an adaptation.

Opsomming

Spesies aangepas vir koue temperature moet volgens die kontroversiële Metaboliese koue aanpassings-hipotese ’n hoër rustende metaboliese tempo of 'n gewysigde metaboliese tempo-temperatuur verhouding hê in vergelyking met hul gematigde eweknieë. Hierdie intra-spesifieke studie verskaf ’n nuwe eksperimentele assessering wat die Metaboliese koue aanpassings-hipotese toets in vier *Drosophila melanogaster* bevolkings, versprei oor verskeie breedtegrade van oostelike Australië. Hier toets ek spesifiek die voorspelling dat bevolkings van hoër breedtegrade (kouer omgewingstemperature) ’n verhoogde rustende metaboliese tempo of 'n laer aktiveringsenergie sal toon, wat 'n verandering in die metaboliese tempo-temperatuur verhouding se sensitiwiteit kan weerspieël. Om te bepaal of akklimatisering ’n invloed op die metaboliese tempo van die bevolkings het, is die verskillende bevolkings in die laboratorium onder algemene toestande grootgemaak waarna hulle vir 5 dae by ’n stabiele 18°C en 25°C geakklimatiseer is, asook by fluktuerende temperature van 13-22°C en 20-30°C. Metaboliese tempo is bepaal by 6 verskillende toetstemperature (10°C, 15°C, 20°C, 25°C 30°C en 35°C). Nadat beide massa en aktiwiteit in ag geneem is, was daar geen bevolking wat konsekwent ’n hoër rustende metaboliese tempo by enige van die akklimatiserings -temperature getoon het nie. In twee populasies, is ’n effens laer aktiveringsenergie verkry by 25°C stabiele akklimatisering, maar die dalende aktiveringsenergie het nie met ’n toename in breedtegraad verband gehou nie. Geen ander verskille in aktiveringsenergie is waargeneem nie. Hierdie studie kom dus tot die gevolgtrekking dat daar geen bewys van die Metaboliese koue aanpassings-hipotese onder bevolkings van *D. melanogaster* in Australië is nie. Dit kan moontlik toegeskryf word aan die gebrek aan konsekwente koue temperature in die hoër breedtegrade van die Suidelike Halfrond wat moontlik so 'n aanpassing sou kon aanvoer.

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following persons:

My supervisor, Prof. John Terblanche. Thank you for your patience, motivation and valued advice. Your support and encouragement were endless, and it was a great privilege to work with you. Thank you for this project and for believing in my abilities.

My co-supervisors, Prof. Steven Chown and Dr. Carla Sgrò. Thank you for granting me the opportunity to travel to and work in your esteemed laboratories at Monash University in Melbourne. It is an experience I will never forget. Your advice on the project is highly valued.

Fiona Beasley and Ian Aitkenhead. Thank you for all your help and for all the laughs while I was working at Monash. I would have been totally lost without you- literally, I had no idea where to go. You both gave me remarkable guidance and made my stay a very joyful one.

My parents, Margaret and Frank. Thanks for letting me follow my heart and supporting me all the way. I am a tremendously lucky kid and I will never forget it.

And finally, my partner Quihen. Thank you for all the early morning coffees, midnight teas, and everything you do for me in-between them.

Preface

This thesis is presented as a compilation of 1 chapter and is written according to the style of the journal Evolution.

Table of Contents

Chapter 1

1.1 Introduction

Metabolism is the foundation to all life, reflecting an organism's net ATP consumption rate, with metabolic rate (MR) representing the cost to an individual to maintain life in a specific habitat. Evidence suggests that variation in MR, among or between individuals, population or species, reflects the optimization of life sustaining biological processes to maximise an individual's performance in a particular thermal environment (Addo-Bediako, Chown, & Gaston, 2002; White, Alton, & Frappell, 2012). Despite the significance of MR, its evolution remains unclear, with many hypotheses regarding MR variation shrouded in controversy and conflicting findings, a prime example being the hypothesis of metabolic cold adaptation (MCA).

Originally proposed by Fox (Fox, 1936) MCA (also known as temperature compensation (Hazel & Prosser, 1974) or Krogh's rule (Gaston et al., 2009)) suggests that ectotherms originating from cold climates (typically high latitude or altitude) possess higher MR in comparison to their warmer counter parts when compared at the same test temperature (Fox, 1936; Addo-Bediako, Chown, and Gaston 2002; Terblanche et al. 2009). MCA is said to manifest itself either via cold adapted species displaying an elevated Metabolic Rate Temperature (MR-T) curve at a given temperature (Figure 1), or via altered sensitivity (i.e. steeper/reduced slope) of the MR-T relationship curve (Chown, Haupt, & Sinclair, 2016; Messamah, Kellermann, Malte, Loeschcke, & Overgaard, 2017). A reduced MR-T slope means MR does not decline to the same degree in cold temperatures as it would had the slope been steeper (Chown et al., 2016). Alternatively, an increased slope may allow species to exploit short-term increases in ambient temperature (Terblanche et al., 2009). An altered slope results in an altered activation energy (E_a) of metabolism. MCA is thought to be a compensatory mechanism, allowing those species from cold environments to maintain a high enough MR to support or sustain various key processes such as development and reproduction at lower temperatures- or development over shorter, cooler growing seasons (Addo-Bediako, Chown, & Gaston, 2002; Sømme & Block, 1991; White, Alton, & Frappell, 2012).

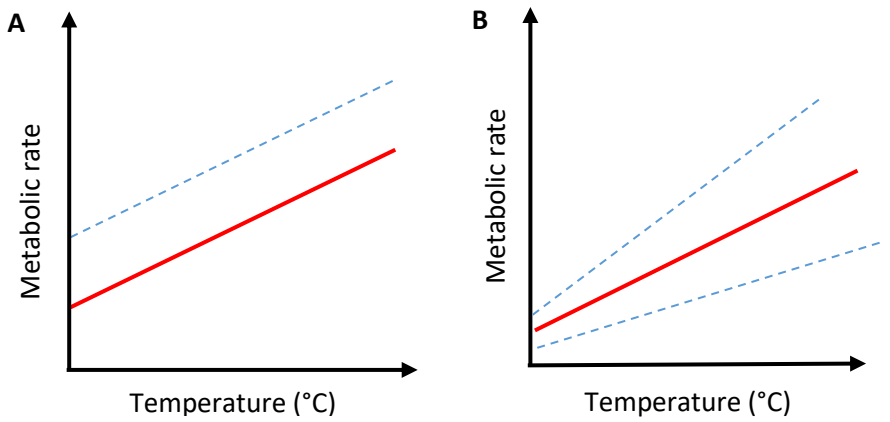


Figure 1: Two of the possible ways metabolic cold adaptation can present itself. A) Cold adapted species (dashed line) have a higher MR at a given temperature when compared to warm adapted species. B) Cold adapted species have an altered (increased or reduced) slope when MR is plotted temperature- meaning they have a lower/higher metabolic action energy when compared to warm adapted species.

This hypotheses has proven to be highly contentious in nature (Clarke, 2003), as some studies (both inter and intraspecific) have found evidence of MCA in arthropods (Addo-Bediako et al., 2002; Block & Young, 1978; Chappell, 1983; Terblanche, Clusella-Trullas, Deere, Van Vuuren, & Chown, 2009; White et al., 2012) while others have not (Alton, Condon, White, & Angilletta, 2017; Berrigan & Partridge, 1997; Clarke, 1993; Lardies, Bacigalupe, & Bozinovic, 2004; Messamah et al., 2017; Nylund, 1991; Oikawa, Mori, & Kimura, 2006). One of the most comprehensive interspecific studies of MCA to date is the global-scale analysis of the resting metabolic rates of 346 species of ectotherms conducted by Addo-Bediako, Chown, and Gaston (2002). Perhaps the most significant result was that when controlling for the effects of trial temperature, wing status, experimental method and body mass, species from colder environments tended to have a higher RMR at 25°C. This effect was weak but was found in both Northern and Southern Hemisphere and ultimately gave support to MCA, even though it was well acknowledged that in some species MCA is less pronounced or absent. When testing for MCA in the MR-T relationship, Northern Hemisphere species showed an increase in slope that was not observed in Southern Hemisphere species. This finding was thought to be a result of decreased seasonality amongst higher Southern Hemisphere latitudes when compared to Northern Hemisphere counterparts (Danks, 1999). It was also noted that data from the Southern Hemisphere were lacking. A smaller scale investigation into MCA by Messamah et al. (2017) looked at 65 species of Drosophilidae (vinegar flies), originating from sub-arctic to tropical environments. The study sought to control for environmental confounding effects by rearing flies under common garden conditions prior to MR estimation. MR was recorded at two test temperatures (10 and 20°C) using intermittent closed system respirometry. The study found no evidence to support MCA in drosphilids - a result which was previously observed in similar studies (see Alton et al., 2017; Oikawa, Mori, and Kimura, 2006).

While support for MCA may vary among taxa and studies, many of these studies can be subject to criticism due in part to the experimental design employed. There are many factors that need to be controlled and accounted for in order to accurately test for MCA- including activity, body size, thermal history, feeding status, and age or life-stage of the study species (Hodkinson, 2003). Activity data in particular is often lacking, with many recordings of MR being included under the assumption that the test subjects were at rest or that their activity did not contribute significantly to MR. But in assuming that spontaneous activity is negligible, those measurements may not reflect the true relationship between MR and thermal effects. For example, Halsey et al. (2015) demonstrated this issue nicely by recording both the MR and activity of three invertebrate ectotherm species across a range of temperature conditions, and found that temperature effects on total MR deviate from estimates based on resting MR alone since spontaneous activity varied greatly within and among the three species. When quantifying MCA, studies have been criticized for their use of extrapolation of the MR-T curve using a standard temperature coefficient (Q10) of 2. Q10 is the rate of change in resting metabolic rate (RMR) as a result of a temperature

increase of 10 °C (Halsey, Matthews, Rezende, Chauvaud, & Robson, 2015). It has been argued that Q10 does not always increase linearly and therefore this extrapolation would confound results (Hodkinson, 2003). It has also been suggested that a low activation energy of metabolism (E_a -calculated from the gradient of the Arrhenius plot of log metabolism against 1/temperature and another method for assigning thermal sensitivity of reaction rates) is the best way to evaluate cold adaptation, rather than Q10 (Hodkinson, 2003; Sømme & Block, 1991). The experimental respirometry method can also introduce substantial bias, as studies using closed-system respirometry methods have often given higher estimates of standard metabolic rate than flow-through respirometry (Lighton & Fielden, 1995).

One other methodological issue that has emerged recently is the lack of accounting for thermal history effects or phenotypic plasticity of metabolic rate (Chown & Terblanche, 2006; Irlich, Terblanche, Blackburn, & Chown, 2009). When these effects have been accounted for typically they would use stable controlled thermal conditions. However it is increasingly well appreciated that the magnitude of the variability in the thermal acclimation treatment might be a significant feature of the organism's response (Alton et al., 2017;Colinet, Renault, & Roussel, 2017; Sgrò, Terblanche, & Hoffmann, 2016). Temperature fluctuations occur naturally throughout environments at different time-scales (Sheldon & Dillon, 2016) and MR-T curves may vary following exposures to constant or fluctuating temperatures (Chown, Haupt, & Sinclair, 2016; Foray, Desouhant, & Gibert, 2014). The vast majority of investigations into MCA make use of constant temperatures alone, disregarding the effects of thermal variability that could alter the shape of the MR-T curve in the natural environment. It has also been noted that there is a lack of intraspecific studies amongst MCA investigations, despite these studies being widely accepted as the best way to evaluate evolutionary changes (Lardies, Bacigalupe, & Bozinovic, 2004). Finally, Hodkinson (2003) has suggested that MCA may constitute cold adapted species displaying relatively higher RMR only at low temperatures alone, as well as an increase in locomotor activity at low temperatures, enabling species quickly to take advantage of small rises in their environmental temperature.

This study builds on to the previous investigations of MCA in *Drosophila* but takes an inter-population comparative experimental approach. Here I aim to compare populations of *Drosophila melanogaster* found across a latitudinal cline of eastern Australia (Figure 2) to assess if higher latitude (i.e. colder environmental temperatures) populations have a higher RMR, or an altered MR-T relationship. Thermal traits of *D. melanogaster* found in Australia have shown clinal patterns in previous studies(Hoffmann, Anderson, & Hallas, 2002). Although these patterns have varied according to assay method (Sgrò et al., 2010) . The study measures and accounts for three of most commonly accepted factors that affect MR: size, activity and ambient temperature (Terblanche, Clusella-Trullas, Deere, Van Vuuren, & Chown, 2009). This study also reduces the need for extrapolation to determine MR-T relationships by taking measurements across a broad range of biologically relevant test temperatures (10, 15, 20, 25, 30, and 35°C). In addition to testing whether there is a latitudinal gradient associated pattern of MCA, the

study also investigates whether acclimating those populations to a stable environment (either 25°C or 18°C) or a fluctuating environment (20-30°C or 13-23°C) has any significant effect on MR or the MR-T relationship. This will give novel insights in to whether a: A) evolutionary adaptation to a colder environment (i.e. population comparison after rearing in a common environment); B) acclimation to a colder environment or; C) a temporal variation in temperature (seasonality) perhaps selects for an elevated MR. If MCA is present it is expected that the highest latitude population will have the highest RMR/ significantly different E_a, or populations acclimated to colder temperatures will have a higher RMR/significantly different E_a. The study further aims to explain if responses of MR to temperature that were detected might have been driven by variation in locomotor activity at low temperatures.

1.2 Methods

1.2.1 Fly stocks and maintenance

Populations of *D. melanogaster* were collected from four study sites located along the eastern coast of Australia: Innisfail (Ins; -17.53, 146.03); Rockhampton (Rhp; -23.15, 150.70); Ballina (Bln; -28.76, 153.53) and Melbourne (Mlb; -37.73, 145.45) (Figure 2). Mass-reared populations were established in the laboratory prior to the experiment by collecting field-inseminated females (30-50 per location) and making isofemale lines. From these lines, ten virgin males and ten virgin females from each location were used as parents for F1. Fly generations were between F19 and F22 at the time of the experiment.

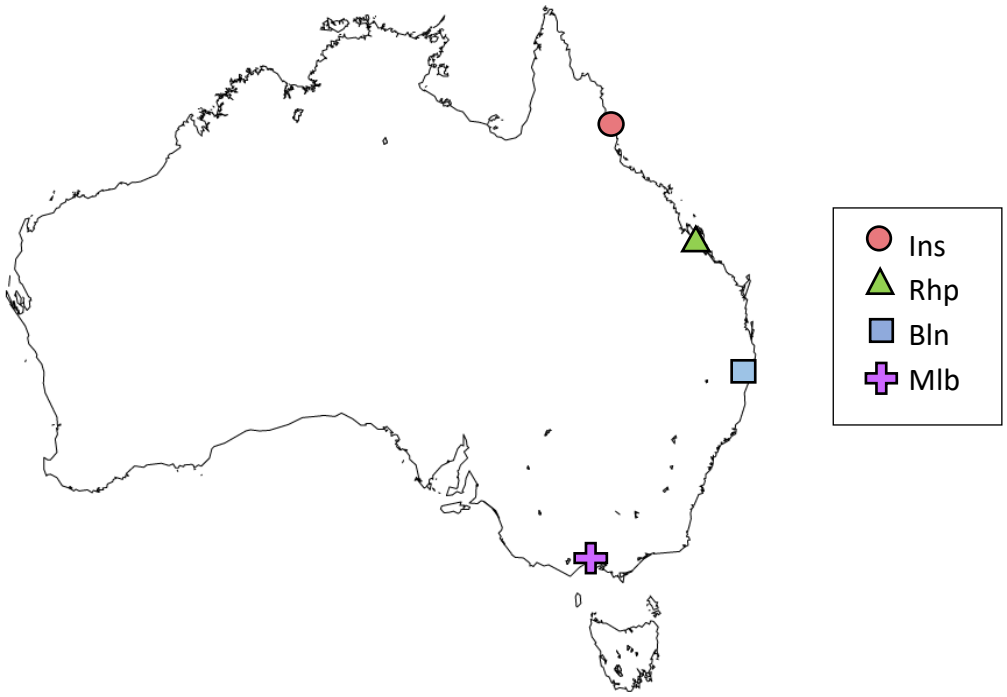


Figure 2: Four study sites across a latitudinal cline of eastern Australia where populations *of D. melanogaster* were collected prior to the experiment.

The populations were maintained on a potato-yeast-dextrose-agar medium at 25 °C (12L:12D light cycle). Populations were maintained under laboratory culture at a size of at least 1000 flies per generation before use in experiment. Laboratory adaptation for this species is considered negligible, as Berrigan and Partridge (1997) compared freshly caught populations of *D. melanogaster* vs. 100 generations of laboratory adapted individuals and showed a < 10% difference in metabolic rate.

1.2.2 Experimental Design

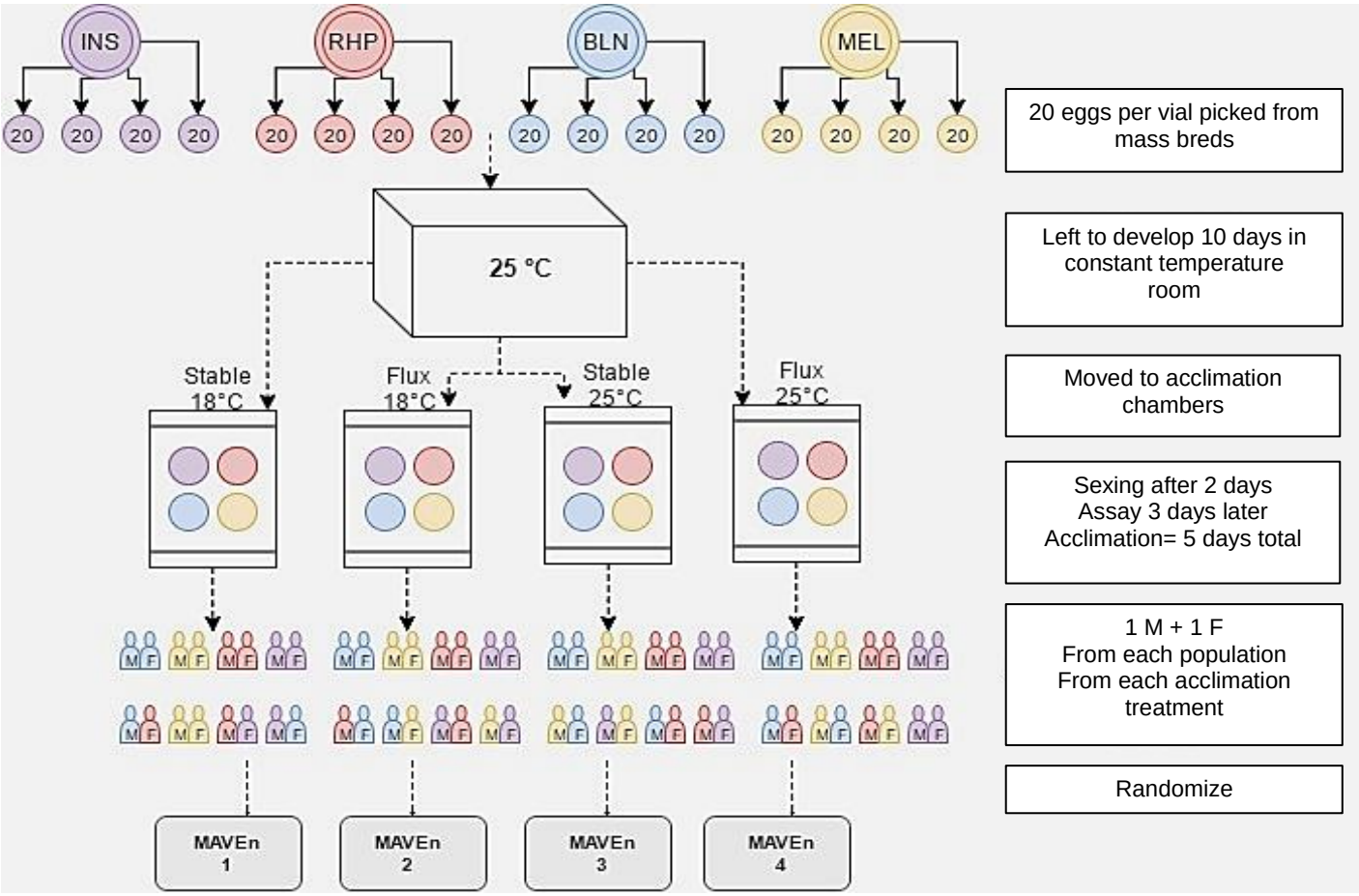


Figure 3: Schematic of the experimental design starting from picking eggs and ending in placing flies into respirometry equipment

The experimental schematic is presented in Figure 3. Experimental flies were identical in age, food accessibility, and acclimation duration. To achieve this, new eggs were laid every day throughout the experiment from the parent generation. Two sets of parent generations were used, with parents over the age of 10 days old being discarded. Eighty eggs from each study site population were picked per day and laid in 40ml vials containing 7ml of potato-yeast-dextrose-agar medium. Four vials per population were used, with each containing twenty eggs to prevent potential overcrowding effects. The vials were placed in a 25°C constant temperature room (12L:12D light cycle) for 10 days so that flies could fully eclose as adults. Newly emerged adults were then transferred to vials containing fresh medium, and one vial from each population was placed in each of the four acclimation chambers. The acclimation chambers were set at stable 25°C (T_{accS25}); stable 18°C (T_{accS18}); fluctuating 25°C (T_{accF25}); fluctuating 18°C (T_{accF18}). Fluctuating temperatures cycled up 5°C then down 5°C from the set temperature, changing by 1°C every hour. Temperature was monitored with an onboard thermometer and was verified by Thermochron DS1921 i-

button dataloggers. Flies were acclimated for two complete days, then anesthetized using CO_2 and separated by sex (using a maximum of 5 minutes sedation time). Males and females were placed into separate vials containing fresh medium, and returned to their respective acclimation chambers for a further 3 days to recover from sedation and limit any influence of CO_2 on the metabolic rate estimate (Colinet & Renault, 2012). After a total of 5 days of acclimation, one male and one female were randomly selected from each population, from each of the acclimation chambers. Flies were individually placed into 1ml TriKinetics activity tubes that were marked to ensure all flies had similar amounts of room for activity. The tubes were then randomly placed in one of the four MAVEn (Sable Systems International, Las Vegas, Nevada) systems for measurement of metabolic rate and activity simultaneously.

1.2.3 Metabolic rate measurements

Carbon dioxide production (VCO_2) was used as a proxy for metabolic rate and was measured using 4 Sable Systems International (SSI) multiple animal versatile energetics systems (MAVEn (Sable Systems International, Las Vegas, Nevada)) - each attached to a separate Li-Cor 7000 CO_2/H_2O infrared gas analyzer (Figure 4).

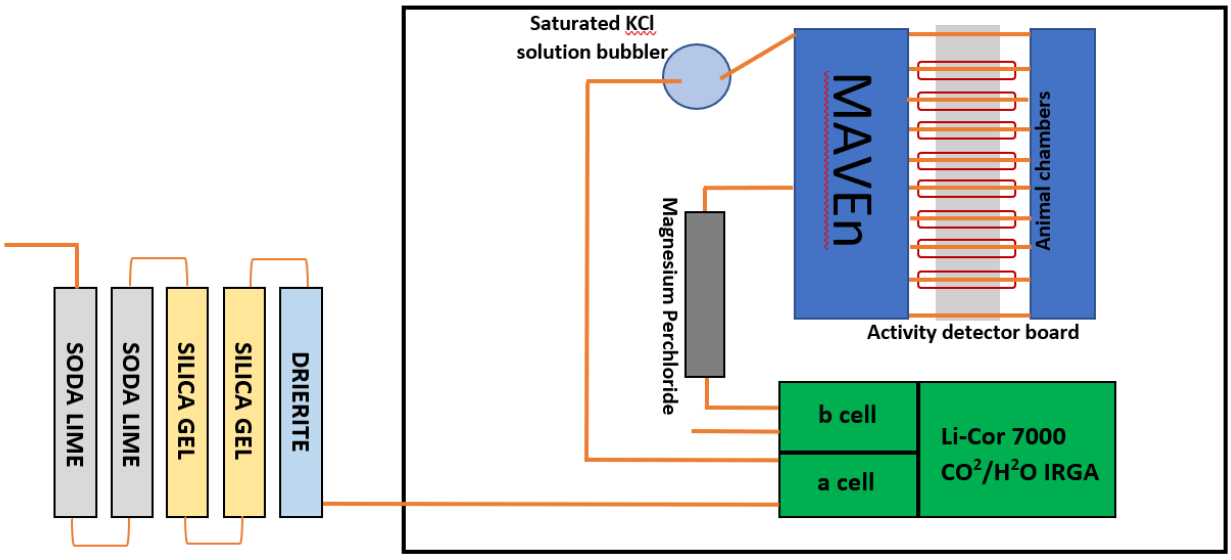


Figure 4: Simplified diagram of a MAVEn and respirometry unit and gas flow. Gas flow is indicated with a red line.

For each MAVEn system, a compressed air flow was directed through a series of 5 scrubber columns (2 soda lime, 2 silica gel, 1 dririte) to ensure a CO_2, H_2O free air stream. The airstream was first directed to "A" cell of the Li-Cor 7000 where an initial reading of CO_2 was taken, then directed to the input of a MAVEn system which housed 8 individual fly respirometry tubes. The gas flow was directed over each of these 8 chambers, with the MAVEn system sequentially measuring each individual fly by directing the flow from

one chamber back to the Li-Cor "B" cell where a second measurement of CO_2 was taken. The difference between the cell A and cell B measurements was taken as VCO_2 and recorded in ppm. Each fly was measured twice for a period of 5 minutes (10 minutes in total). A 5-minute baseline recording was taken every 4 chambers (20 min) to account for drift in the Li-Cor 7000 over the course of the experiment, which was typically minimal or non-existent. Activity of each *D. melanogaster* was measured simultaneously using infrared light detectors in the MAVEn activity board. Activity was a unitless measurement, and was detected as fly movement changes in the infrared light field above each detector. Flow rate was set by the MAVEn system and held constant at 35 ml/minute throughout all experiments using a mass flow control valve which is built into MAVEn. Li-Cor 7000s were 0 calibrated (baselined) at 20°C prior to each experimental trial commencing. A full two-point calibration was undertaken (H_2O, followed by CO_2) with a span gas of 12 ppm CO_2 on a weekly basis for each Li-Cor analyzer. Calibration accuracy was <1% of 12ppm. Though only 8 channels of the MAVEn system were used in the experiment, MAVEn is a 16 channel system. The 8 channels not in use were filled with empty 1ml Trikinetics activity tubes, but these channels were not selected for measurement.

The system was housed in a climate control cabinet (Panasonic MIR 352H-PE, Japan) which controlled temperature throughout the experiment. Metabolic rate was measured at 6 set-point temperatures randomized in the following order 20°C, 15°C, 10°C, 25°C, 35°C, 30°C. Each temperature ran for 2.5 hours. Temperature was randomized to prevent short-term acclimation responses or potential cumulative stress of sequentially increasing or decreasing temperatures. Temperature within the respirometry experiment is measured and recorded using the on-board MAVEn thermometer. To prevent potential effects of desiccation over the course of each experimental trial, the airstream was bubbled through a saturate potassium chloride solution which humidified each chamber at 82-86% RH. On the out flow (exhaust port) of the MAVEn system, the airstream was directed through a magnesium perchloride scrubber to remove water vapor from the airstream prior to the second CO_2 measurement being taken.

A total of 32 flies were tested per assay, and 12 assays were completed, making n=12. After the assay had run to completion, any deceased individuals were recorded. Flies were then placed in a -80°C freezer for a minimum of 4 hours. The body weight of each individual fly was recorded (Sartorius Cubis®, 0.0001 mg resolution). Flies were removed from the freezer and individually weighed within 45 seconds in order to prevent water loss. The flies are then dehydrated at 50°C for 24 hours and dry mass is recorded.

1.2.4 Data acquisition, treatment and analysis

System control and data acquisition were all performed by the MAVEn systems SD card. Data were processed using Expedata (SSI) software, and were lag and baseline corrected for each experimental temperature, though the latter effects were typically minimal. Data were extracted via Expedata (SSI) using

a macro coded to retrieve the most level, nadir, and zenith 30 seconds of activity and the corresponding CO^2 (ppm) from each 5 minute recording. The most level data windows were selected for all subsequent analysis and the corresponding average activity level of flies at each trial temperature also extracted for the same data interval. Statistical analysis of activity effects were ran on zenith data (most active 30 seconds) and found no significant difference between the two data sets. The mean activity and corresponding mean CO_2 for the most level data was used for analysis. CO_2 ppm was converted using standard temperature and pressure flow rate to ml/h ((reading in µm/m after baseline and lag correction / 1000000) X flow rate (ml/min) X 60(min/h)) which was taken as an estimate of MR. Dry body mass was used in place of fresh mass as some individuals had died before weighing, and desiccation would have confounded fresh body mass data.

All statistical analyses were executed using R v3.3.3 in R studio v1.1.383, with all graphs being constructed with the package ggplot2. Statistical analyses to test variation in MR were performed using linear mixed-effects model (lmer found in package lme4). Initial models included all fixed variables (Mass:Activity:Population:Acclimation:Sex) and individual as a random factor. Initial models showed that both mass and activity influenced MR. To adjust for the effects of mass and activity on MR, residuals from MR and mass + activity were used in subsequent analyses. These residuals were taken to represent resting metabolic rate and are referred to as RMR hereafter. After adjusting for mass, sex had no effect on RMR and was excluded from subsequent analyses. Variance in RMR was tested using lmer and included temperature as a continuous variable, acclimation and population as fixed effects, with individual as a random variable. After testing for all interactions, the best fit model was determined via model selection (package MuMIN). A post-hoc test between populations at each test temperature within each acclimation group was done using lmer. A population with a t value >2 was considered to have an effect.

Statistical analyses to test variation in body mass, activity at 10°C, and E_a were performed with generalized linear models using a gaussian distribution. To obtain the minimum adequate model (MAM), model simplification by stepwise deletion of non-significant interaction terms was used from the full saturated model and inspection of the AIC was used to determine the best quality model (Crawley, 2007). E_a was calculated for each population at each acclimation taking the slope of residuals obtained from a GLM of the natural logarithm (Ln) of MR and the variables Mb and activity, plotted against 1/KT, where K is Boltzmann's constant (8.617 x 10-5 eV/K) and T is temperature in Kelvin. Figures for E_a and activity are presented as box-and-whisker plots for maximum transparency (Weissgerber, Milic, Winham, & Garovic, 2015).

Data used to investigate temperature variance (monthly minimum and maximum) at the study sites was extracted from Worldclim2 (Fick & Hijmans, 2017), which is a database containing average monthly climate data for minimum, mean, and maximum temperature and for precipitation for the years 1970-2000.

1.3 Results

1.3.1 Mass differences between populations and acclimation conditions

When investigating whether mass differences between study populations and test acclimations were a driving force of MR variation, it was found that individuals from acclimation stable 25 °C and population Ins were significantly larger, but only by a small amount (<2%) (Appendix S1).

1.3.2 MCA: A higher RMR

After model selection (Table 1), test temperature was found to be the only significant effect on RMR, with a positive effect increasing with increasing temperature (Table 2, Figure 5). No populations and no acclimation conditions had significantly different RMRs, nor did any of their interactions, so they were excluded from the model. A post hoc analysis was performed to investigate the differences in RMR between populations at each test temperature within each acclimation group. It was found that when acclimated at stable 25°C the highest latitude population (Mlb) had a significantly higher RMR at 15°C (t=2.255, Figure 5A). No other population had a significantly different RMR (*t-value* > 2) over any test temperature for any acclimation.

1.3.3 MCA: A lower E_a

When acclimated at stable 25°C, Mlb and Ins had a significantly lower E_a (Table 3; Figure 6). Acclimating populations to a fluctuating 25°C resulted in a small but significantly lower E_a (although this effect is not seen across all populations; Table 3). While population had no effect on E_a, it was retained in the final model due to the higher order interaction of population x acclimation (Stable 25°C) being significant.

1.3.4 Changes in activity level at low temperatures

Differences in activity levels at low temperatures (10°C and 15°C) were explored and it was found when acclimated to fluctuating 25°C, Mlb had a significantly higher level of activity at 10°C than all other populations (Figure 7; Appendix S1). No other differences in activity between populations were found.

1.3.5 Variance in temperatures at study sites

A temperature cline is seen correlating with latitude, with lower latitudes having higher mean monthly maximum and minimum temperatures (Figure 8). All sites have maximum temperatures above the reproduction threshold throughout the year, and the higher latitude sites reach temperatures that are considered optimal for *D. melanogaster* (+-20°C) from October through March.

Table 1: Model selection output of all models investigating possible effects on RMR of four *D. melanogaster* populations following acclimation at constant and fluctuating temperatures. Models contained fixed effects of test temperature (T_{test}), population (P), acclimation (T_{acc}) and their interactions on RMR, with individual (Ind) as a random effect. Best fit model is listed first (bold) with lowest AIC.

Model	Intercept	df	Log Likelihood	AIC	Delta (Δ)	Weight
T_{test} + (1|Ind)	**-0.00022**	**4**	**16595.18**	**-33174.4**	0.00	1
T_{test} + T_{acc} + (1|Ind)	-0.00049	7	16449.72	-32885.4	50.72	0
T_{test} + P + (1|Ind)	-0.00048	7	16449.32	-32884.6	51.52	0
T_{test} * T_{acc} + (1|Ind)	-0.00050	10	16421.34	-32822.7	113.48	0
T_{test} * P + (1|Ind)	-0.00050	10	16417.80	-32815.6	120.56	0
T_{test} * T_{acc} + P + (1|Ind)	-0.00049	13	16398.60	-32771.2	164.96	0
T_{test} + T_{acc} * P + (1|Ind)	-0.00048	19	16369.74	-32701.5	234.67	0
T_{test} * T_{acc} * P + (1|Ind)	-0.00050	34	16231.18	-32394.4	541.80	0
(1|Ind)	0.00001	3	15313.90	-30621.8	2314.36	0
T_{acc} + (1|Ind)	0.00001	6	15291.49	-30571.0	2365.18	0
P+ (1|Ind)	0.00001	6	15291.10	-30570.2	2365.95	0

Table 2: Summary of the best fit model of the effects on RMR of four *D. melanogaster* populations following acclimation at constant and fluctuating temperatures. Effects at 10°C are retained in the model as a reference.

Effect	Estimate	S.E	*df*	*t*	*P*
T_{test} (°C)					
15	0.00041	0.0001	3166	3.924	0.0000
20	0.00170	0.0001	3170	16.086	0.0001
25	0.00195	0.0001	3190	16.476	0.0000
30	0.00328	0.0001	3166	31.327	0.0000
35	0.00577	0.0001	3166	55.139	0.0000

Table 3: MAM of the effects on the E_a of four *D. melanogaster* populations following acclimation at constant and fluctuating temperatures. Effects at F18 and Bln are retained in the model as reference. Significant effects are highlighted in bold.

Effect		Estimate	S.E	*t*	*P*
T_{acc}	**F25**	**-0.06830**	**0.0219**	**-3.118**	**0.0019**
	S18	-0.04270	0.0222	-1.923	0.0546
	S25	0.01150	0.0219	0.526	0.5992
P	Ins	0.00723	0.0216	0.334	0.7383
	Mlb	0.01270	0.0218	0.584	0.5592
	Rhp	0.00082	0.0235	0.035	0.9722
T_{accF25} X	P_{Ins}	-0.00233	0.0307	-0.076	0.9395
	P_{Mlb}	0.04330	0.0300	1.442	0.1494
	P_{Rhp}	-0.01020	0.0327	-0.313	0.7544
T_{accS18} X	P_{Ins}	-0.00205	0.0310	-0.066	0.9472
	P_{Mlb}	0.00280	0.0304	0.092	0.9266
	P_{Rhp}	0.03970	0.0323	1.23	0.2189
T_{accS25} X	**P_{Ins}**	**-0.08800**	**0.0303**	**-2.909**	**0.0037**
	P_{Mlb}	**-0.10100**	**0.0308**	**-3.292**	**0.0010**
	P_{Rhp}	0.00001	0.0318	0	0.9996

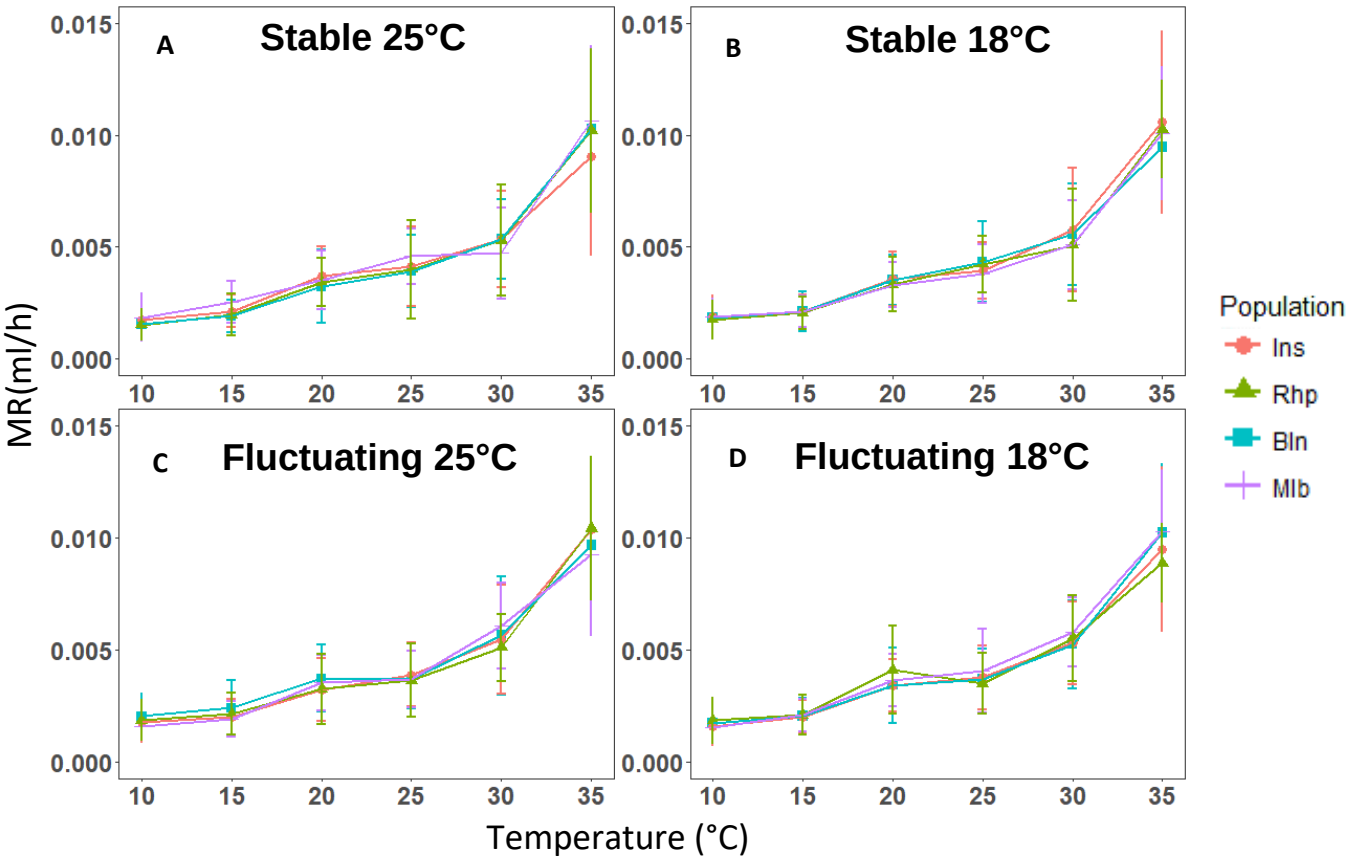


Figure 5: Mean unadjusted MR of four *D. melanogaster* populations following acclimation to A) Stable 25°C; B) Stable 18°C; C) Fluctuating 25°C; D) Fluctuating 18°C.

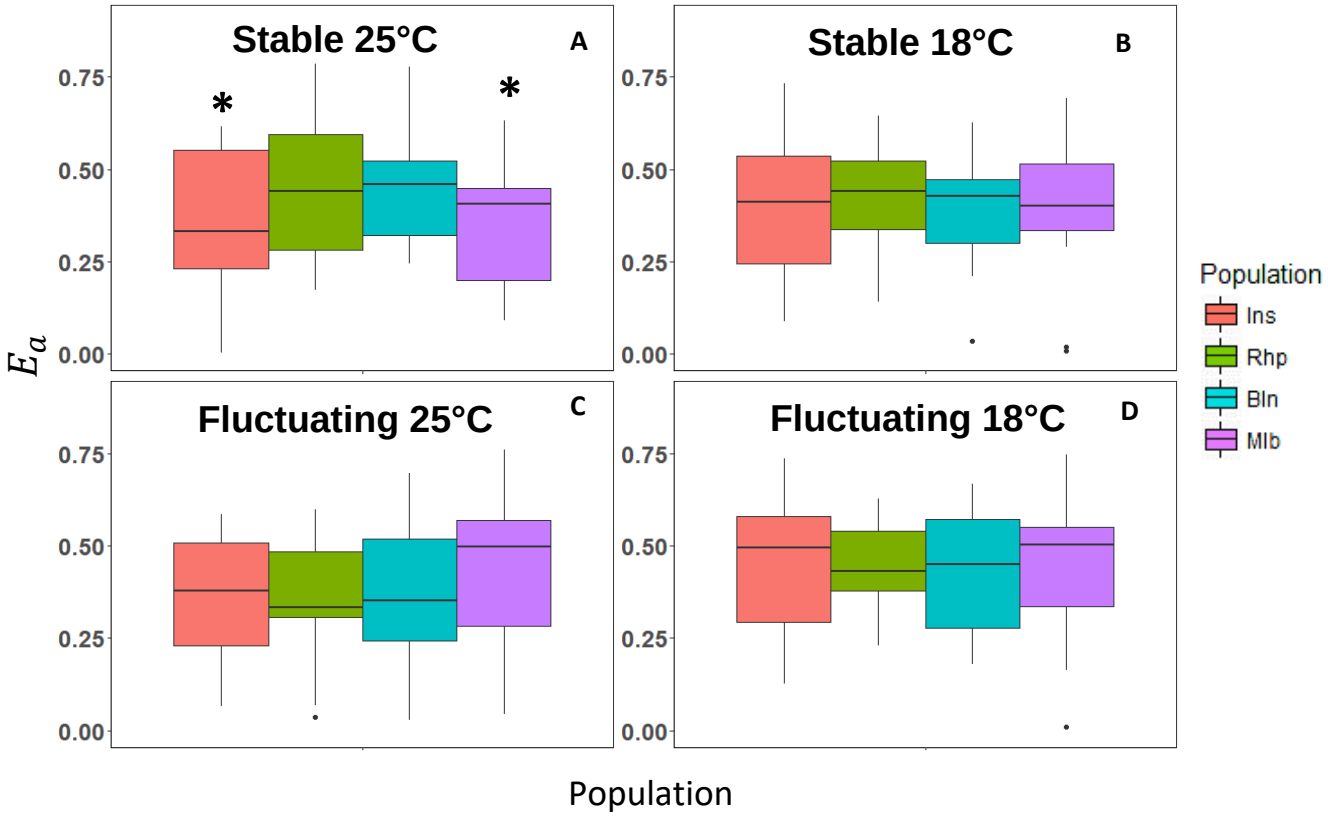


Figure 6: Changes in E_a of four *D. melanogaster* populations following acclimation at A) Stable 25°C; B) Stable 18°C; C) Fluctuating 25°C; D) Fluctuating 18°C. Significant results have be marked with *.

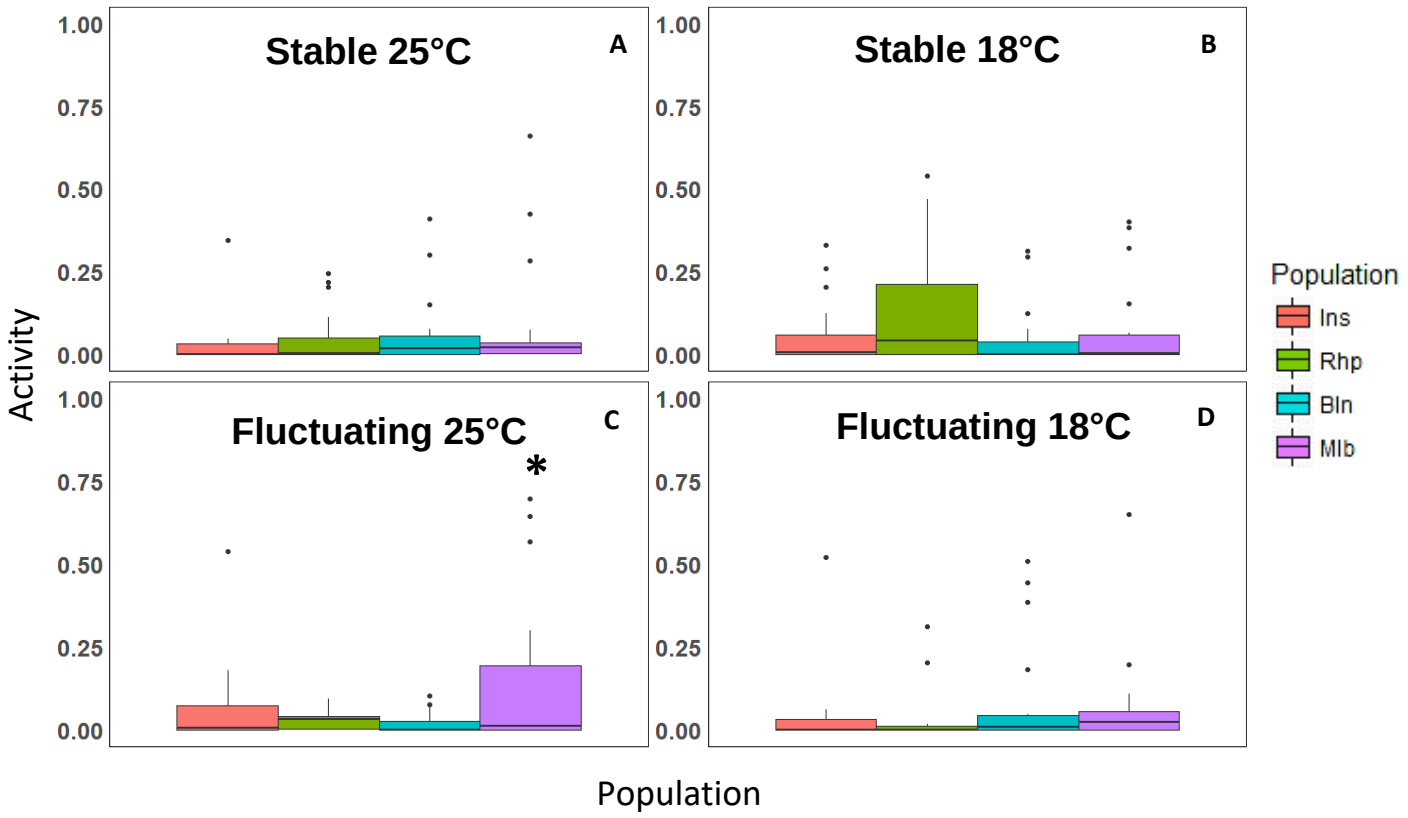


Figure 7: Changes in activity level of four *D. melanogaster* populations at 10°C following acclimation at A) Stable 25°C; B) Stable 18°C; C) Fluctuating 25°C; D) Fluctuating 18°C. Significant results have be marked with *.

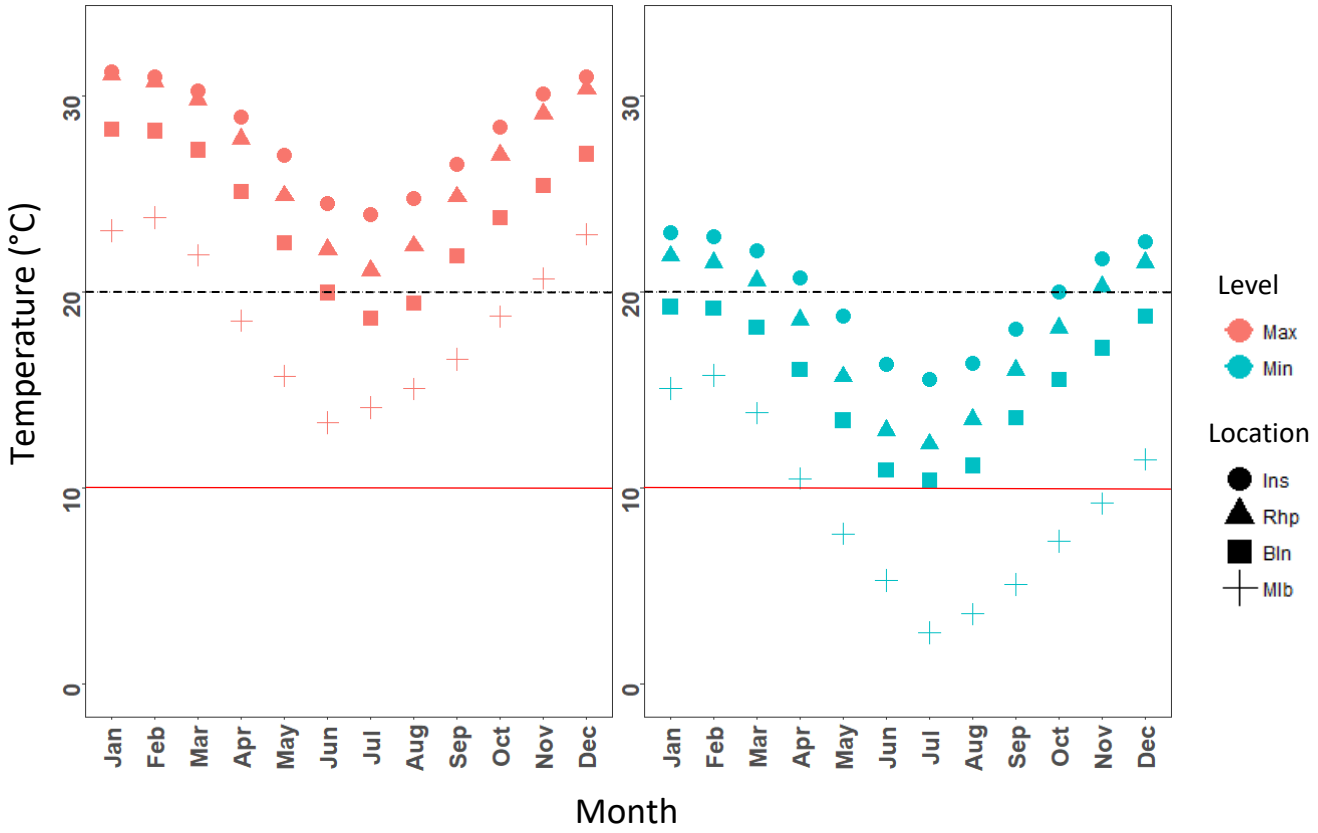


Figure 8: Average monthly maximum (A) and minimum (B) temperatures of the four study sites where populations of *D. melanogaster* were sampled. Lower temperature threshold for reproduction of *D. melanogaster* (red line) as well as an optimal rearing temperature (dash lined) have been annotated.

1.4 Discussion

When investigating whether MCA is present in the form of a higher RMR, I found that no population maintained a significantly higher RMR following any of the four acclimations. When I examined each test temperature within each acclimation in isolation, a significant result was found following acclimation at a stable 25°C, where the highest latitude population (Mlb) had a significantly higher RMR at 15°C. Had Mlb shown significantly higher RMR at low temperatures across other acclimations, this result may have supported the notion by Hodkinson (2003) that MCA might constitute an elevated RMR at low temperatures, but as no other significant differences were found, this does not seem to be the case. This result also demonstrates the importance of testing for MCA over a range of temperatures, as extrapolation of RMR at another temperature may well be misleading. The significant difference seen at stable 25°C but not at the other acclimation conditions is most likely due to variation in thermal reaction norms among populations. Fluctuating and constant temperatures are known to change the shape of thermal reaction norms in ectotherms owing to Jensen's inequality (Foray, Desouhant, & Gibert, 2014; Niehaus, Angilletta, Sears, Franklin, & Wilson, 2012). While the difference in shape of the MR-T curve was not large enough to cause a significant difference in RMR for the other acclimations, at stable 25°C the variance was large enough to see a significant difference for population Mlb- but only at one test temperature. Support for MCA also failed when testing for a lower E_a. While populations Mlb and Ins did show a significantly lower E_a when acclimated to a stable 25°C, the difference was small (<10%) and not seen across all acclimation groups. Furthermore, while Mlb is the highest latitude (coldest) population, Ins is the lowest (warmest), inferring there is no correlation between latitude and E_a. This suggests that the variation in E_a at this acclimation is due to factors other than MCA.

When testing for support for MCA in the form of higher levels of activity at low temperatures, it was noted that flies acclimated to a fluctuating 25°C, Mlb had significantly higher levels of activity at 10°C. While it has been hypothesized that higher latitude ectotherms might show increased plasticity when compared to tropical counterparts (Chown & Terblanche, 2006; but see discussions in Seebacher, White, & Franklin, 2015), no patterns in thermal plasticity (measured as the difference between basal and hardened thermal tolerance) along the same latitudinal gradient in Australia were found for *Drosophila simulans* (van Heerwaarden, Lee, Overgaard, & Sgrò, 2014). This result instead may reflect innate genetic variation in behaviour which manifested as activity differences among populations. For example, an increase in activity could indicate that when acclimated to a fluctuating 25°C, Mlb displays a greater capacity to seek out warmer microclimates during cold periods. Regardless, such a proposal would require closer scrutiny but may be a useful area to explore in future. It must also be noted that activity detected in my experiment is perhaps limited due to the relatively small size of the tube, and so observed activity (and its contribution to

total MR) may not be reflected as such in the field where animals can take short or long-distance flight and explore their environment more extensively.

The lack of support for MCA in drosophilids either at the population or species-level is not entirely unusual and this result is thus in agreement with several previous investigations (Alton, Condon, White, & Angilletta, 2017; Messamah, Kellermann, Malte, Loeschcke, & Overgaard, 2017; Oikawa, Mori, & Kimura, 2006). This study contradicts the large scale MCA pattern found for ectotherms (Addo-Bediako, Chown, & Gaston, 2002), and instead gives support to the notion that there is a lack of MCA in the Southern Hemisphere- adding further controversy to the hypothesis. Additionally, the study demonstrated that there is very little variation in MR-T relationships among the populations, even after acclimation to constant and fluctuating temperatures. The acclimation conditions in this study were however, applied only to adults post-eclosion, and for a period of only five days. As all populations were reared at a constant 25°C, thermal responses that would have perhaps been present during development in the field may have been mitigated. It would be of interest to investigate whether these acclimation conditions induce a change in the MR-T relationship had the populations been exposed to them during development or for a longer period of time. A related study was conducted to determine if *D. melanogaster* evolving at a constant 16°C, constant 25°C, or fluctuations between 16 and 25°C saw any changes in MR-T, and found no difference between treatments (Alton et al. 2017). However, no comparisons were made between populations living along a latitudinal cline. In caterpillars of a sub-Antarctic moth (*Pringleophaga marioni*) tested for variation in MR and MR-T following constant and fluctuating thermal conditions also found little impact among populations, as well as no effect of latitude on temperature-corrected metabolic rate when compared with caterpillars of 13 other Lepidopteran species (Chown, Haupt, & Sinclair, 2016). The lack of variation in MR-T relationships is found through-out terrestrial ectotherms (Gunderson & Stillman, 2015; Seebacher, White, & Franklin, 2015) but results have varied for some groups (Terblanche, Clusella-Trullas, Deere, Van Vuuren, & Chown, 2009).

The lack of MCA detected in these *D. melanogaster* populations in Australia is somewhat unsurprising considering the highest latitude population (Mlb) still has ambient temperatures over 20°C for nearly half the year (November- March). Egg-to-adult viability in *D. melanogaster* is limited to 10-32°C (Petavy, David, Gibert, & Moreteau, 2001) and male fertility is restricted to 12°C - 30°(Chakir, Chafik, Moreteau, Gibert, & David, 2002). Ambient temperatures around 20°C would comfortably allow populations to survive. An increase in MR is costly to organisms- especially at warmer temperatures (Clarke, 1993; 2003), so this increase would only be beneficial if faster growth was necessary to thrive in short, cool growing seasons. For this reason, it has been hypothesized that MCA is more likely to occur in species where their habitats consist of low temperatures throughout the growing season- which occurs less frequently at high latitudes in Southern Hemisphere in comparison to the North (Addo-Bediako, Chown, and Gaston 2002; Chown and Gaston 1999). Drosophilids may also not be the ideal study organism to test

for MCA as their generation time is very short. Insect with short generation times are likely to exploit reasonably high microhabitat temperatures to complete their life cycles, and so would not need to endure the cost of evolving a consistently higher metabolic rate (Chown & Gaston, 1999).

In conclusion, this study failed to find support for MCA in four *D. melanogaster* populations spanning across a large latitudinal cline in Australia. Higher latitude populations did not display consistently higher rates of RMR at most test temperatures, and while there were significantly lower E_a found for one acclimation group, the populations in which the slightly lower E_a were present did not correlate with increasing latitude. The relationship between metabolic rate and temperature is just one piece of the complex puzzle that is metabolic evolution. There are many other factors that alter with season and latitude which could drive variations in metabolic rates, including resource availability and growth rates. But as it stands, it seems one piece of that puzzle is falling in place- temperature variation does not appear to drive metabolic adaptation in the *D. melanogaster* of Australia – or at least not in the adult flies. Given the persistent variation in behaviour detected in the activity assays among some populations it may be worthwhile in future exploring the genetic components of behaviour and thermal or diurnal activity patterns, as this may be a significant avenue for temperature compensation along latitudinal clines (Huey & Pascual, 2009). Nevertheless, few studies have tested for MCA using a whole-lifetime energy budget and to fully integrate development and activity trade-offs within and across stages and *D. melanogaster* may therefore prove to be a powerful model for such an approach in future.

References

Addo-Bediako, A., Chown, S. L., & Gaston, K. J. (2002). Metabolic cold adaptation in insects: a large scale perspective. *Functional Ecology*, *16*(3), 332–338.

Alton, L. A., Condon, C., White, C. R., & Angilletta, M. J. (2017). Colder environments did not select for a faster metabolism during experimental evolution of *Drosophila melanogaster*. *Evolution*, *71*(1), 145–152.

Berrigan, D., & Partridge, L. (1997). Influence of temperature and activity on the metabolic rate of adult *Drosophila melanogaster*. *Comparative Biochemistry and Physiology Part A: Physiology*, *118*, 1301–1307.

Block, W., & Young, S. . R. (1978). Metabolic adaptations of Antarctic terrestrial micro-arthropods. *Comparative Biochemistry and Physiology a-Physiology*, *61*, 363–368.

Chakir, M., Chafik, A., Moreteau, B., Gibert, P., & David, J. R. (2002). Male sterility thermal thresholds in Drosophila: *D. simulans* appears more cold-adapted than its sibling D. melanogaster. *Genetica*, *114*(2), 195–205.

Chappell, M. A. (1983). Metabolism and thermoregulation in desert and montane grasshoppers. *Oecologia*, *56*(1), 126–131.

Chown, S. L., & Gaston, K. J. (1999). Exploring links between physiology and ecology at macro-scales: The role of respiratory metabolism in insects. *Biological Reviews*, *74*(1), 87–120.

Chown, S. L., Haupt, T. M., & Sinclair, B. J. (2016). Similar metabolic rate-temperature relationships after acclimation at constant and fluctuating temperatures in caterpillars of a sub-Antarctic moth. *Journal of Insect Physiology*, *85*, 10–16.

Chown, S. L., & Terblanche, J. S. (2006). Physiological diversity in insects: ecological and evolutionary contexts. *Advances in Insect Physiology*, *33*, 50–152.

Clarke, A. (1993). Seasonal acclimatization and latitudinal compensation in metabolism : Do They Exist ? *Functional Ecology*, *7*(2), 139–149.

Clarke, A. (2003). Costs and consequences of evolutionary temperature adaptation. *Trends in Ecology and Evolution*, *18*(11), 573–581.

Colinet, H., & Renault, D. (2012). Metabolic effects of CO2 anaesthesia in *Drosophila melanogaster*. *Biology Letters*, *8*(6), 1050–1054.

Colinet, H., Renault, D., & Roussel, D. (2017). Cold acclimation allows Drosophila flies to maintain mitochondrial functioning under cold stress. *Insect Biochemistry and Molecular Biology*, *80*, 52–60.

Crawley, M. (2007). *The R book*. New York: Wiley.

Danks, H. V. (1999). Life cycles in polar arthropods - flexible or programmed? *European Journal of Entomology*, *96*, 83– 102.

Fick, S. E., & Hijmans, R. J. (2017). WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *International Journal of Climatology, 37(*12*),* 4302-4315.

Foray, V., Desouhant, E., & Gibert, P. (2014). The impact of thermal fluctuations on reaction norms in specialist and generalist parasitic wasps. *Functional Ecology*, *28*(2), 411–423.

Fox, H. M. (1936). The activity and metabolism of poikilothermic animals in different latitudes. *Proceedings of the Zoological Society of London*, *106A*, 945–955.

Gaston, K. J., Chown, S. L., Calosi, P., Bernardo, J., Bilton, D. T., Clarke, A., … van Kleunen, M. (2009). Macrophysiology: A Conceptual Reunification. *The American Naturalist*, *174*(5), 595–612.

Gunderson, A. R., & Stillman, J. H. (2015). Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1808), 20150401–20150401.

Halsey, L. G., Matthews, P. G. D., Rezende, E. L., Chauvaud, L., & Robson, A. A. (2015). The interactions between temperature and activity levels in driving metabolic rate: theory, with empirical validation from contrasting ectotherms. *Oecologi*a, *177*, 1117–1129.

Hazel, J. R., & Prosser, C. L. (1974). Molecular mechanisms of temperature compensation in poikilotherms. *Physiological Reviews*, *54*(3), 620 -677.

Hodkinson, I. D. (2003). Metabolic cold adaptation in arthropods: A smaller-scale perspective. *Functional Ecology*, *17*, 562–567.

Hoffmann, A., Anderson, A., & Hallas, R. (2002). Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecology Letters* , *5*, 614-618.

Huey, R. B., & Pascual, M. (2009). Partial thermoregulatory compensation by a rapidly evolving invasive species along a latitudinal cline. *Ecology*, *90(*7), 1715–1720.

Irlich, U. M., Terblanche, J. S., Blackburn, T. M., & Chown, S. L. (2009). Insect rate-temperature relationships: environmental variation and the metabolic theory of ecology. *The American Naturalist*, *174*(6), 819–835.

Lardies, M. a, Bacigalupe, L. D., & Bozinovic, F. (2004). Testing the metabolic cold adaptation hypothesis: an intraspecific latutudinal comparison in the common woodlouse. *Evolutionary Ecology Research*, *6*, 567–578.

Lighton, J. R. B., & Fielden, L. J. (1995). Mass scaling of standard metabolism in ticks: A valid case of low metabolic rates in sit-and-wait strategists. *Physiological and Biochemical Zoology*, *68*(1), 43–62.

Messamah, B., Kellermann, V., Malte, H., Loeschcke, V., & Overgaard, J. (2017). Metabolic cold adaptation contributes little to the interspecific variation in metabolic rates of 65 species of Drosophilidae. *Journal of Insect Physiology*, *98*, 309–316 .

Niehaus, A. C., Angilletta, M. J., Sears, M. W., Franklin, C. E., & Wilson, R. S. (2012). Predicting the physiological performance of ectotherms in fluctuating thermal environments. *Journal of Experimental Biology*, *215*(4), 694–701.

Nylund, L. (1991). Metabolic Rates of *Calathus-Melanocephalus* (L) (Coleoptera, Carabidae) from Alpine and Lowland Habitats (Jeloy and Finse, Norway and Drenthe, the Netherlands). *Comparative Biochemistry and Physiology a-Physiology*, *100*(4), 853–862.

Oikawa, A., Mori, N., & Kimura, M. T. (2006). Comparison of oxygen consumption in drosophilid flies from different climates. *Entomological Science, 9,* 347–354.

Petavy, G., David, J. R., Gibert, P., & Moreteau, B. (2001). Viability and rate of development at different temperatures in Drosophila: a comparison of constant and alternating thermal regimes. *Journal of Thermal Biology*, *26*(1), 29–39.

Seebacher, F., White, C. R., & Franklin, C. E. (2015). Seebacher F, White CR, Franklin CE. Physiological plasticity increases resilience of ectothermic animals to climate change. *Nature Climate Change*, *5*, 51–66.

Sgrò, C. M., Overgaard, J., Kristensen, T. N., Mitchell, K. A., Cockerell, F. E., & Hoffmann, A. A. (2010). A comprehensive assessment of geographic variation in heat tolerance and hardening capacity in populations of *Drosophila melanogaster* from Eastern Australia. *Journal of Evolutionary Biology*, *23*(11), 2484–2493.

Sgrò, C. M., Terblanche, J. S., & Hoffmann, A. A. (2016). What can plasticity contribute to insect responses to climate change? *Annual Review of Entomology*, *61*(1), 433–451.

Sheldon, K. S., & Dillon, M. E. (2016). Beyond the mean: Biological impacts of cryptic temperature change. *Integrative and Comparative Biology*, *56*(1), 110–119.

Sømme L., Block W. (1991) Adaptations to alpine and polar environments in insects and other terrestrial arthropods. In: Lee R.E., Denlinger D.L. (eds) *Insects at Low Temperature*. Springer, Boston, MA

Terblanche, J. S., Clusella-Trullas, S., Deere, J. A., Van Vuuren, B. J., & Chown, S. L. (2009). Directional evolution of the slope of the metabolic rate–temperature relationship is correlated with climate. *Physiological and Biochemical Zoology*, *82*(5), 495-503.

van Heerwaarden, B., Lee, R. F. H., Overgaard, J., & Sgrò, C. M. (2014). No patterns in thermal plasticity along a latitudinal gradient in *Drosophila simulans* from eastern Australia. *Journal of Evolutionary Biology*, *27*(11), 2541–2553.

Weissgerber, T. L., Milic, N. M., Winham, S. J., & Garovic, V. D. (2015). Beyond bar and line graphs: Time for a new data presentation paradigm. *PLoS Biology*, *13*(4), 1–10.

White, C. R., Alton, L. A., & Frappell, P. B. (2012). Metabolic cold adaptation in fishes occurs at the level of whole animal, mitochondria and enzyme. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1734), 1740–1747.

Appendix S1

Table 1: MAM of the effects of population and acclimation on body mass of four *D. melanogaster* populations following acclimation at constant and fluctuating temperatures. Effects at Fluctuating 18°C are retained in the model as a reference. Significant effects are highlighted in bold.

Effect		Estimate	S. E	*t*	*P*
Sex	**F**	**0.03312**	**0.0056**	**5.830**	**0.0006**
T_{acc}	F25	0.00048	0.0080	0.060	0.9522
	S18	-0.01325	0.0080	-1.647	0.0998
	S25	**0.01832**	**0.0080**	**2.289**	**0.0222**
P	**Ins**	**0.01784**	**0.0079**	**2.245**	**0.0249**
	Mlb	0.01130	0.0078	1.444	0.1489
	Rhp	0.00424	0.0082	0.517	0.6055

Table 2: MAM of the effects of population and acclimation on mean activity at 10°C and 15°C of four *D. melanogaster* populations following acclimation at constant and fluctuating temperatures. Significant effects are highlighted in bold.

Effect			Estimate	S.E	*t*	*P*
T_{test10}						
	T_{acc}	F25	-0.07173	0.0504	-1.423	0.1558
		S18	-0.04103	0.0510	-0.804	0.4220
		S25	-0.03041	0.0504	-0.603	0.5468
	P	Ins	-0.05627	0.0498	-1.129	0.2598
		Mlb	-0.02800	0.0498	-0.562	0.5747
		Rhp	-0.05316	0.0552	-0.962	0.3370
	T_{accF25} X	P_{Ins}	0.09903	0.0708	1.397	0.1634
	T_{accS18} X	P_{Ins}	0.06678	0.0713	0.936	0.3499
	T_{accS25} X	P_{Ins}	0.02452	0.0708	0.346	0.7296
	T_{accF25} X	**P_{Mlb}**	**0.14988**	**0.0691**	**2.168**	**0.0310**
	T_{accS18} X	P_{Mlb}	0.13329	0.0708	1.882	0.0609
	T_{accS25} X	P_{Mlb}	0.05838	0.0708	0.824	0.4108
	T_{accF25} X	P_{Rhp}	0.06025	0.0765	0.787	0.4320
	T_{accS18} X	P_{Rhp}	0.13072	0.0757	1.726	0.0855
	T_{accS25} X	P_{Rhp}	0.04382	0.0748	0.586	0.5585
	T_{acc}	F25	-0.06089	0.0403	-1.510	0.1320
T_{test15}		S18	-0.03044	0.0407	-0.746	0.4560
		S25	-0.04276	0.0398	-1.072	0.2847
	P	Ins	-0.05304	0.0395	-1.343	0.1803
		Mlb	-0.04480	0.0398	-1.123	0.2623
		Rhp	-0.07264	0.0431	-1.684	0.0932
	T_{accF25} X	P_{Ins}	0.08319	0.0564	1.474	0.1416
	T_{accS18} X	P_{Ins}	0.05383	0.0567	0.948	0.3438
	T_{accS25} X	P_{Ins}	0.03533	0.0553	0.638	0.5238
	T_{accF25} X	P_{Mlb}	0.10489	0.0553	1.897	0.0588
	T_{accS18} X	P_{Mlb}	0.11002	0.0559	1.967	0.0501
	T_{accS25} X	P_{Mlb}	0.08490	0.0564	1.505	0.1334
	T_{accF25} X	P_{Rhp}	0.07387	0.0599	1.232	0.2189
	T_{accS18} X	P_{Rhp}	0.09251	0.0593	1.559	0.1201
	T_{accS25} X	P_{Rhp}	0.09040	0.0583	1.549	0.1224